The Penny Pusher: A Cellular Model of Lens Growth

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Submitted: November 7, 2014

Accepted: December 1, 2014

Citation: Shi Y, De Maria A, Lubura S, Šikić H, Bassnett S. The Penny Pusher: a cellular model of lens growth. *Invest Ophthalmol Vis Sci.* 2015;56:799–809. DOI:10.1167/iovs.14-16028

PURPOSE. The mechanisms that regulate the number of cells in the lens and, therefore, its size and shape are unknown. We examined the dynamic relationship between proliferative behavior in the epithelial layer and macroscopic lens growth.

METHODS. The distribution of S-phase cells across the epithelium was visualized by confocal microscopy and cell populations were determined from orthographic projections of the lens surface.

RESULTS. The number of S-phase cells in the mouse lens epithelium fell exponentially, to an asymptotic value of approximately 200 cells by 6 months. Mitosis became increasingly restricted to a 300-μm-wide swath of equatorial epithelium, the germinative zone (GZ), within which two peaks in labeling index were detected. Postnatally, the cell population increased to approximately 50,000 cells at 4 weeks of age. Thereafter, the number of cells declined, despite continued growth in lens dimensions. This apparently paradoxical observation was explained by a time-dependent increase in the surface area of cells at all locations. The cell biological measurements were incorporated into a physical model, the Penny Pusher. In this simple model, cells were considered to be of a single type, the proliferative behavior of which depended solely on latitude. Simulations using the Penny Pusher predicted the emergence of cell clones and were in good agreement with data obtained from earlier lineage-tracing studies.

CONCLUSIONS. The Penny Pusher, a simple stochastic model, offers a useful conceptual framework for the investigation of lens growth mechanisms and provides a plausible alternative to growth models that postulate the existence of lens stem cells.

Keywords: epithelium, growth, model

The number of cells in epithelial tissues generally reflects a balance between cell proliferation and cell differentiation. How these processes are coordinated to generate structures of appropriate size and shape is not well understood in any system. In the lens of the eye, cell number must be regulated with particular precision if light is to be focused sharply on the retina.

The lens is composed of two types of cells: epithelial cells and fiber cells. Epithelial cells, arranged in a mono-layer, cover the anterior surface of the lens. At the edge of the epithelium, near the lens equator, epithelial cells differentiate continuously into fiber cells,¹ a process that involves, among other things, a striking (10²- to 10³-fold) increase in cell length. Newly differentiated fiber cells are deposited on the surface of the preexisting fiber mass. As a result, the lens grows continuously throughout life, albeit at a reduced rate in later years.² There is no cell turnover in the fiber cell compartment. Consequently, fiber cells that differentiated early in life are retained near the center of the adult lens, whereas the superficial tissue layers are populated by newly differentiated fibers.

It is well established that cell proliferation in the lens is restricted to the epithelium.³ Under normal circumstances, expression of cyclin-dependent kinase inhibitors p27^{kip1} and p57^{kip2} ensures that fiber cells and the outermost rim of epithelial cells (a region called the transition zone [TZ]) are postmitotic.⁴ The rate of epithelial cell division varies with both the age of the lens (being highest in young animals)^{5,6} and

latitudinal position within the epithelial sheet.⁷ Early studies using tritiated thymidine demonstrated that S-phase cells are most numerous in the germinative zone (GZ) of the epithelium, a loosely defined swath of cells encircling the lens, just above the equator.^{8,9} Daughter cells produced by mitosis in the GZ are eventually displaced (or migrate) to the equator, whereupon they differentiate into fiber cells after exposure to growth factors in the vitreous humor.¹⁰

The basic organization of the lens epithelium is well established, but several issues remain unresolved. It is unclear, for example, whether the lens epithelium contains a contingent of tissue stem cells analogous to those found in the nearby corneal limbus and, if so, where, in the apparently uniform field of epithelial cells, the stem cell niche might reside. Lineage-tracing studies¹¹ suggest that clones of epithelial cells emerge as cells traverse the GZ and are incorporated into the fiber cell mass, but current models do not account for epithelial cell clonality.⁷ The deposition of new fiber cells causes a steady increase in the volume and surface area of the lens. Presumably, additional epithelial cells are required to populate the expanding anterior surface. It is not known whether those cells are also provided by the GZ. If the GZ is the source of additional epithelial cells, it implies that cells with two distinct fates are produced in this zone: fiber progenitors, which move toward the equator and are eventually incorporated into the body of the lens, and epithelial progenitors, which move in the opposite direction, facilitating expansion of the epithelial sheet.

Whether cells with differing fates are produced in the GZ and, if so, how they are sorted and dispatched in opposite directions is not known. Finally, although a number of studies have analyzed the proliferative behavior of cells in the epithelium, few have examined cell death rates. The presence of apoptotic cells has been noted in the developing lens¹² but the rate of apoptosis in healthy adult lenses is unknown. The lack of information on this important parameter has made it difficult to adequately model lens epithelial cell dynamics.

In this study, we examined in detail how epithelial cell proliferative rates varied with latitudinal position and age in the mouse lens. The analysis provided insights into the substructure of the GZ and the complex relationship between cell proliferation rate and cell area on the anterior surface of the growing lens. Data were incorporated into a “Penny Pusher” model of lens growth that captured the essential clonal behavior observed in lineage-tracing studies.

METHODS

Animals

Wild-type mice (C57BL/6J) and transgenic green fluorescent protein (GFP)-expressing mice (TgN[GFPU]5NAGY),¹³ were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were killed by CO₂ inhalation. Eyes were enucleated and lenses were either imaged immediately or processed for 5-ethynyl-2'-deoxyuridine (EdU) labeling, as described below. To control for possible sex differences, only male animals were used in this study. All procedures were approved by the Washington University Animal Studies Committee and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Epithelial Cell Census, EdU-Labeling Index, and Cell Size

Cells in S-phase of the cell cycle were identified following incorporation of EdU (Invitrogen, Carlsbad, CA, USA), as described.^{14,15} The EdU was administered as a single intraperitoneal injection. Injections were made at the same time each day (10–11:00 AM local time) to control for circadian variations in cell cycle. Mice were generally killed 1 hour after EdU injection, but, to examine the migration of recently divided cells, some animals were examined 1 week to 2 months after the initial EdU injection. The EdU-positive cell nuclei were visualized using Click-iT (Invitrogen) chemistry with Draq5 (Cell Signaling Technology, Danvers, MA, USA) as a nuclear counterstain.

The EdU/Draq5-labeled lenses were imaged by confocal microscopy (Zeiss 510 LSM; Carl Zeiss, Thornwood, NY, USA).¹⁴ The number of EdU-labeled nuclei and total number of cells in the epithelium were determined from orthographic maximum intensity projections of the anterior and equatorial lens surfaces. Groups of EdU-positive nuclei formed recognizable patterns visible from both anterior and equatorial aspects, allowing anterior and equatorial projections of the lens surface to be brought into registration.¹⁴ The number of Draq5-labeled nuclei was then determined using automated image analysis, as described.¹⁴ At the edge of the epithelium, cell nuclei become organized into meridional rows. Meridional row cells have already begun to differentiate into fiber cells and were therefore excluded from the epithelial cell census.

Preliminary studies confirmed that proliferation index varied with latitudinal position in the lens epithelium. To quantify this effect, we generated en face maximum projections of the equatorial region. A 20 × 200-μm measurement

quadrat was defined on the projected image, oriented such that the long axis of the quadrat was parallel to the lens equator. The number of EdU-labeled nuclei and the total number of Draq5-labeled nuclei within the quadrat were calculated. These data also were used to calculate average cell size. The quadrat was then stepped in 20-μm increments away from the equator. Labeling index, cell number, density, and size were calculated at each latitudinal location.

Surface Area and Volume Measurements

The surface area and volume of the lens were calculated by measuring the polar and equatorial lens radii, assuming the lens to be an oblate spheroid. Calculations were performed with an online calculator (planetcalc.com/149/). The epithelium was assumed to cover half of the total surface area. The average surface area of cells located at different latitudinal positions in the lens epithelium was determined from orthographic projections of confocal image stacks from Draq5-stained lenses. To examine changes in cell volume and apical surface area of individual living cells, TgN(GFPU)5Nagy mice were used. In those animals, GFP is expressed in a mosaic pattern, allowing individual cells to be optically isolated and imaged at high resolution in the intact tissue.¹⁶ Image stacks were deconvolved (Huygens essential; SVI, Hilversum, The Netherlands) using an experimentally determined point spread function, as described.¹⁷ Cell volume was measured using the Huygens object analyzer subroutine. The area of the apical cell membrane was determined by scrolling through the optical sections and selecting the confocal image corresponding to the apical membrane. The area of the membrane was then determined by using software supplied with the confocal microscope.

Cell Death

Apoptotic cells were detected by incubating isolated lenses in tissue culture medium (Dulbecco's modified Eagle's medium-F12; Gibco Life Technologies, Grand Island, NY, USA) containing 5 μM fluorogenic caspase substrate (CellEvent caspase-3/7 detection reagent; Invitrogen) for 30 minutes at 37°C. In this assay, caspase activation results in the cleavage of a DEVD (Aspartic acid-Glutamic acid-Valine-Aspartic acid) tetrapeptide sequence within the parent molecule and subsequent release of a DNA-binding dye. Apoptotic cells are then identified by the presence of fluorescent nuclei. Some lenses were preincubated for 24 hours in 1 μM staurosporine (a broad-spectrum kinase inhibitor; Sigma-Aldrich Corp., St. Louis, MO, USA) to induce apoptosis, as a positive control for the assay.

RESULTS

The mouse lens forms between day 10 (E10) and E11 of embryonic development.¹⁸ By E18.5, the first stage examined here, the lens had a volume of 0.17 mm³ and a surface area of 1.51 mm². By 46 months of age (the oldest stage examined), lens volume had increased more than 50-fold and the surface area more than 14-fold compared with E18.5 values. As shown in Figure 1, the growth of the lens was initially rapid but slowed to a near imperceptible rate at later stages. In lenses from young mice, proliferating (EdU-labeled) cells were numerous and distributed throughout the epithelium, although even at E18.5, labeled cells were less numerous in the central epithelium than the equatorial epithelium. By 4 weeks of age, proliferating cells were observed only rarely in the central

FIGURE 1. Growth of the mouse lens and distribution of proliferating cells. Maximum intensity orthographic projections of segments of the anterior lens hemisphere were used to produce this composite image. Note that lens growth is rapid during embryonic and early postnatal development but slows considerably at later time points. Proliferating cells (EdU-labeled; green) are numerous in young lenses, where they are distributed throughout the epithelium. At later stages, fewer proliferating cells are observed and these are increasingly restricted to the equatorial epithelium. The *inset* shows a higher-magnification view of the central epithelium. Note also that the cells are more densely packed in the epithelium of the younger lenses. Nuclei (red) are counterstained with Draq5. Scale bars: 250 μm and 100 μm (*inset*).

epithelium and mitosis was increasingly restricted to a broad swath of the equatorial epithelium, the GZ.

Quantitative analysis (Fig. 2) revealed that the number of S-phase cells in the lens fell sharply over the first 6 months of life but stabilized, thereafter, at a value of approximately 200 cells per lens. The decline was well described by a single exponential decay of the form $Y = Y_0 + ae^{-bx}$, where $Y_0 = 193$, $a = 1296$, and $b = 0.1843$. Previous studies reported that S-phase lasts approximately 12 hours in the mouse lens epithelium,¹⁹ suggesting that, even in aged animals, approximately 400 cells per day are generated by epithelial cell mitosis.

As development proceeded, proliferating cells became increasingly restricted to the peripheral epithelium near the

lens equator (Fig. 1). This region has conventionally been termed the GZ. To better define the topology of this zone, we imaged the spatial distribution of EdU-labeled cells across the spherical anterior surface of intact lenses. In this fashion, the variation of labeling index with distance from the lens equator was computed (Fig. 3). The labeling index profile was determined in lenses from 2-week-old to 46-month-old mice. At all ages examined, most labeled cells were located in an approximately 300- μm -wide band, encircling the lens approximately 100 μm anterior to the equator. This region corresponded to the GZ identified in earlier studies. Unexpectedly, the distribution of EdU-labeled cells within the GZ was bimodal, although it was difficult to discern the two maxima in individual confocal images, due to the relatively low labeling

FIGURE 2. The number of S-phase cells per lens decays to an asymptotic value of approximately 200 labeled cells. Parameter values represent best fit to $Y = Y_0 + ae^{-bx}$. Data represent mean $\pm$ SD of three independent measurements at each time point.

index (Fig. 3A); however, when multiple images were superimposed (Fig. 3B), two distinct peaks were apparent. In lenses from 8-week-old mice, the labeling maxima were located 150 μ m (peak a) and 280 μ m (peak b) from the equator (Figs. 3B, 3C).

The edge of the epithelial sheet is demarcated by the presence of meridional rows (MR) (Fig. 3A). In the MR, the nuclei of differentiating fiber cells become radially aligned, as fiber cells begin the initial stages of elongation. Interposed between the GZ and the MR was a 100- μ m-wide region of the epithelium in which EdU-labeled cells were observed only very rarely. In 8-week-old mice, this zone was 8 to 10 cells wide. In keeping with earlier naming conventions, we refer to this postmitotic region of the epithelium as the TZ. Although most EdU-labeled cells were located in the GZ, proliferative cells were also detected (albeit at lower frequency) in a zone bordering the GZ and extending a farther 400 μ m toward the anterior pole of the lens. The existence of an equivalent region, with reduced levels of cell proliferation, was noted previously in the rat lens.²⁰ Here, we refer to this band of cells as the *pregerminative zone* (PGZ). The labeling index in the PGZ

was 5- to 10-fold lower than the peak labeling index in the GZ (Fig. 3C). The region of the epithelium between the anterior margin of the PGZ and the apical pole of the lens was called the *central zone* (CZ). In adult mice, the CZ corresponded approximately to the region of the lens epithelium visible through the dilated pupil. During early development, S-phase cells were commonly detected in the CZ (Fig. 1), but, by 2 months of age, EdU-labeled cells were no longer detected in this region. The arc length from the lens equator to the center of the epithelium in an 8-week-old mouse was approximately 1600 μ m. Therefore, the TZ (100- μ m wide), GZ (300- μ m wide), and PGZ (400- μ m wide) together accounted for approximately 50% of the arc length and a correspondingly larger proportion of the anterior surface area of the lens.

The distribution of labeled cells within the proliferation zones of the lens was similar at all ages (Fig. 4), although the labeling index was uniformly reduced in older animals. At each age, most EdU-labeled cells were located within the GZ, with a labeling maximum (peak a) located approximately 150 μ m from the edge of the epithelium. The labeling index within peak a declined with age, from more than 7% at 2 weeks of age to less than 3% at 6 months.

In younger lenses (2 weeks to 2 months of age), a second peak (peak b) was evident, located approximately 300 μ m from the epithelial margin. The labeling index was consistently higher in peak a than in peak b. Peak b was more difficult to distinguish in lenses from older (6 to 46 months of age) mice, where the labeling index was reduced. In older samples, peak b was displaced by approximately 50 μ m toward the anterior (position b* in Fig. 4).

The migration/differentiation of lens epithelial cells was visualized at intervals after EdU incorporation (Fig. 5). As expected, immediately after EdU treatment, labeled cells were located primarily in the GZ and, to a lesser extent, the PGZ. One week after EdU treatment, cells were present as labeled pairs, indicating the successful completion of mitosis. In the intervening period, some EdU-labeled cells traversed the TZ and entered the MR. Four weeks after EdU treatment, labeled cells were no longer present in the GZ. Presumably, by that stage, cells had migrated through the GZ, TZ, and MR, becoming incorporated into the underlying fiber cell mass. This notion was supported by volumetric reconstructions identifying EdU-labeled nuclei in the deeper fiber cell layers

FIGURE 3. Variation of EdU-labeling index with distance from the lens equator in 8-week-old mice. (A) The epithelial margin is demarcated by the appearance of MR. EdU-labeled cells (green) are most common in the GZ, although labeled cells are also detected, at lower frequency, in the PGZ. (B) Superimposition of six individual projections reveals two peaks (a and b) of labeling intensity within the GZ. (C) Quantitative analysis confirms the existence of two labeling maxima within the GZ. The EdU-labeling index falls to zero at approximately 800 μ m from the equator. This marks the border between the PGZ and the CZ, the anterior cap of epithelial cells wherein S-phase cells are only rarely observed. Quantitative data represent mean and SD ($n = 6$).

FIGURE 4. Distribution of EdU-labeled cells as a function of age and distance from the lens equator. Data represent mean values of more than six determinations at each age. Error bars have been omitted for clarity but are similar in magnitude to those shown in Figure 3C. Within the GZ, two labeling peaks, *a* and *b*, are apparent. At later stages (>6 months), peak *b* is shifted anteriorly to position *b**.

(Supplementary Fig. S1). Alternatively, if cells underwent multiple rounds of division in the GZ, the EdU label may have been diluted below the detection limit. Similarly, the disappearance of cells from the GZ might reflect significant rates of cell death in this area, although cell death assays suggested that this explanation was unlikely (see Fig. 9). Four weeks after the initial labeling protocol, the only EdU-positive nuclei detected in the epithelium were paired nuclei in the PGZ. Eight weeks after the initial labeling period, paired nuclei persisted in the PGZ. The PGZ cell pairs were generally well separated from other cell pairs, suggesting that the EdU-labeled cells had not undergone a second mitosis in the 8 weeks after the initial cell division.

We described previously a method for obtaining an accurate count of the total number of cells in the mouse lens epithelium.¹⁴ Our approach used orthographic projections of the anterior polar and equatorial regions of the mouse lens, which were brought into registration using the unique constellations of EdU-labeled cells as fiduciary points. In the present study, we used this technique to count the total number of epithelial cells in lenses extracted from 1-week-old to 46-month-old mice (Fig. 6). Over this period, lenses grew in all dimensions (see also Fig. 1) and the area of the epithelium increased more than 2-fold. The number of cells in the lens epithelium increased during early postnatal development, initially paralleling the increase in epithelial surface area. The maximum epithelial cell population (approximately 50,000 cells) was reached by 4 weeks of age. Thereafter, cell numbers declined before stabilizing at approximately 40,000 cells by 10 weeks of age (although there was considerable sample-to-sample variation; see Fig. 6). From 10 weeks of age to 46 months of age, the number of cells in the epithelium remained relatively constant, despite continued expansion of the epithelial surface area.

The observation that growth in the surface area of the epithelium was not accompanied by a commensurate increase in epithelial cell number suggested that the surface area of individual lens cells increased over time. The alternative explanation, that gaps appear between the cells, is not supported by the present study or a plethora of previous histological studies. To test the hypothesis, cell surface area

FIGURE 5. Migration of EdU-labeled cells. Immediately after EdU treatment ($t=0$), labeled cells (green) are located exclusively in the GZ and PGZ. One week ($t=1w$) after EdU treatment, pairs of labeled cells are observed, some of which have traversed the TZ and entered the MR. At later time points ($t=4w, 8w$), labeled cell pairs persist in the PGZ but are not detected in the GZ. Scale bar: 200 μm .

was measured in defined regions of the TZ, GZ, PGZ, and CZ (Fig. 7). In the early postnatal lens epithelium, cells were small and packed tightly, although, even at the first stage examined (postnatal day 3), CZ cells were already somewhat larger than cells in the TZ and GZ. Between P3 and 1 week, there was little change in cell size but, beginning at week 2, the surface area of cells at all locations expanded rapidly. The rate of increase was greatest in cells located at higher latitudes (i.e., closer to the anterior pole of the lens). Thus, between 1 and 4 weeks of age, CZ cells increased 3.3-fold in area, and TZ cells increased 1.8-fold.

To test whether increases in epithelial cell area were accompanied by a corresponding increase in volume, individual living lens cells in the CZ were reconstructed in three dimensions (Fig. 8). For this purpose, TgN[GFPU]5NAGY mice were used. In the TgN[GFPU]5NAGY transgenic strain, 20% to 30% of epithelial cells express GFP, allowing living individual

FIGURE 6. Variation in the epithelial cell population (*filled triangles*) and area of the anterior lens epithelium (*filled circles*) with time. The maximum epithelial cell count (approximately 50,000 cells) is reached by 4 weeks of age, after which the population declines to approximately 40,000 cells, despite the continued expansion in the anterior lens surface area.

cells to be visualized in intact living lenses. The CZ cells increased significantly in size during development (Figs. 8A, 8B). At all ages, the basolateral surface had a complex morphology, making direct quantification difficult. In contrast, the apical membrane formed a contiguous, broadly polygonal surface, the area of which could be readily measured. Individual cell measurements confirmed that the apical surface increased 3- to 4-fold over the first year (Fig. 8C). Most of this increase occurred during the first month of life. The increase in apical surface area, measured *in situ* in individual living cells, was consistent with values based on population averages measured on fixed tissue (Fig. 7). There was also a significant increase in CZ cell volume, from approximately $750 \mu\text{m}^3$ in newborn mice, to approximately $1300 \mu\text{m}^3$ in adults. The relative change in cell volume (<2 -fold) was less than that in cell area (3- to 4-fold), implying that the epithelium thins during development. Measurements of single-cell thickness

(the perpendicular distance between the apical and basal membranes) confirmed this notion (Fig. 8E). Lens epithelial CZ cells in newborn mice were approximately $14 \mu\text{m}$ thick. In adult lenses, CZ cells were approximately $7 \mu\text{m}$ thick. Thus, measurements on living epithelial cells suggested that during development, CZ cells spread, covering a much larger surface and becoming considerably thinner in the process.

The rate of apoptotic cell death in the lens was measured by incubating lenses in fluorogenic caspase substrates. In this assay, activation of caspase 3/7 causes cleavage of a DEVD consensus sequence and release of a DNA-binding fluorescent dye. Thus, apoptotic cells are identified by the presence of fluorescent nuclei. As a positive control for the assay, some lenses were incubated for 24 hours in $1 \mu\text{M}$ staurosporine, a treatment previously shown to induce lens epithelial apoptotic cell death.¹² Staurosporine treatment caused widespread cell death in the epithelium (Fig. 9); however, in untreated samples, cell death was not observed. It is important to note that the entire epithelium was inspected under conditions in which even a single apoptotic cell would have been detected. No instance of cell death was observed anywhere in the epithelium in four independent experiments on six animals.

FIGURE 7. Change in cell surface area as a function of latitudinal position and age. Data represent mean $\pm$ SD ($n \geq 3$). The TZ represents average cell area 0 to $100 \mu\text{m}$ from the equator, GZ represents average cell area 200 to $300 \mu\text{m}$ from the equator, PGZ represents average cell area 400 to $500 \mu\text{m}$ from the equator, and CZ is the average cell area within a $300 \times 300\text{-}\mu\text{m}$ measurement quadrat positioned in the center of the epithelium.

DISCUSSION

Growth Rates

According to Snell's law of refraction, the focal length of a convex lens depends on the radius of curvature of its surfaces. Thus, to provide a sharp image on the retina, the living lens has to be precisely the right size. The number of cells in the epithelium reflects a balance between cell production in the GZ/PGZ and emigration of cells from the epithelium into the fiber compartment. Apoptotic cell death does not appear to be an important factor in regulating the size of the epithelial cell population. Epithelial cells express the full complement of genes necessary for apoptosis,²¹ including the executioner caspases,¹² and studies have shown that apoptosis can be triggered by surgical trauma²² or UVB exposure.²³ However, under normal circumstances, the apoptotic index appears to be extremely low (at least in relation to the proliferation rate) in the adult lens.

FIGURE 8. Changes in the dimensions of individual epithelial cells over time. (A) Volume-rendered image of a living P7 epithelial cell (apical membrane is *outlined*) imaged in the intact epithelium. (B) Similar image of an optically isolated epithelial cell in a 6-month-old lens. Significant, age-related changes occur in apical membrane area (C), volume (D), and cell thickness (E). Data represent mean $\pm$ SD.

Although closely coupled, the processes of cell production and emigration/differentiation appeared to be regulated independently in the lens. Early in postnatal development (<4 weeks of age), the number of cells in the epithelium increased rapidly, broadly paralleling the overall expansion of the epithelial surface. In the absence of cell death, an increase in epithelial cell number suggests that the rate of epithelial cell mitosis exceeded the rate of fiber cell differentiation during this period. Between 1 and 2 months of age, however, this situation reversed transiently and fiber cell differentiation briefly exceeded the rate of epithelial cell proliferation, leading to 20% depletion in the epithelial cell population. From 12 weeks of age onward, the epithelial cell population stabilized at approximately 40,000 cells, a value consistent with previous estimates,^{7,14,24} indicating that the rates of cell production and differentiation were now in equilibrium.

The number of cells generated in the proliferative zones of the epithelium fell exponentially to an asymptotic value of approximately 400 cells per day by 6 months of age. Given the stable epithelial cell population at this age and the absence of

cell death, this implies that newly differentiated fiber cells were also deposited at a rate of approximately 400 cells per day. The addition of a constant number of fiber cells to the surface of a growing lens is expected to result in a modest slowing of the radial growth rate at later ages for geometric reasons. For example, if it is assumed that fiber cells have a uniform cross-sectional area of $20 \mu\text{m}^2$ (corresponding to $10 \times 2 \mu\text{m}$ flattened hexagonal cross section), then addition of 400 newly differentiated fiber cells would increase the area of the equatorial plane by $8000 \mu\text{m}^2$. For an 8-week-old lens (radius approximately $1150 \mu\text{m}$) this would cause the diameter to increase by $2.2 \mu\text{m}$. By comparison, in the 12-month-old lens, which has a radius of approximately $1500 \mu\text{m}$, addition of 400 cells would increase the diameter by only $1.7 \mu\text{m}$. This phenomenon is expected to contribute to the slowed growth observed at later ages (see Fig. 1). Nevertheless, the steady addition of 400 cells per day should have caused a noticeable increase in the equatorial radius of the lens between 6 months of age and 46 months of age. A marked increase was not observed, suggesting that some degree of compaction of the

FIGURE 9. Incidence of apoptotic cell death in the lens epithelium is undetectably low. Lenses were cultured for 24 hours in the presence or absence of 1 μ M staurosporine and then incubated with a fluorogenic caspase substrate to monitor DEVDase activity (*green*). Lens epithelial nuclei in staurosporine-treated samples were fluorescent, whereas those in untreated lenses were not. These data indicate that cell death rates in healthy lens epithelia are undetectably low.

preexisting fiber cell population occurred during this period. Fiber cell compaction is a well-documented phenomenon in aged lenses of humans and other species.^{25,26}

Nature and Organization of the Proliferative Zones

In the adult lens, the epithelium can be subdivided into discrete zones based on the proportion of cells in S-phase of the cell cycle. Cells in the CZ are believed to be arrested in G₀ but can be induced to reenter the cell cycle by mechanical injury.²⁷ Given the undetectably low levels of cell death in this

region, it is likely that the longevity of the CZ cells rivals that of the innermost fiber cells. The TZ cells at the epithelial margin are a postmitotic population. Notch signaling in TZ cells has been shown to regulate expression of cyclin-dependent kinase inhibitors and likely plays an important role in defining the boundary between the epithelial and fiber cell compartments.^{28,29} The PGZ and GZ represent the proliferatively active regions of the epithelium and, therefore, constitute the “growth engine” of the lens. In 8-week-old mice, the combined meridional arc length of the GZ and PGZ, approximately 700 μ m, represents more than 40% of the total distance from the

FIGURE 10. Penny Pusher model. Three frames (days 0, 4.5, and 11) from an animated sequence (see Supplementary Video S1) are shown. This physical model covers approximately 10 degrees of longitude of the equatorial epithelium of the 8-week-old mouse lens. Individual cells are represented by pennies. “Cells” are allowed to divide at the rates shown (PGZ 0.5%, GZ 5.0%, TZ 0%). Cell division is simulated by inserting a penny directly adjacent to a randomly selected cell. Ten white cells are present at the beginning of the experiment. These cells have identical replicative behavior to nonwhite cells and are simply included to allow cell lineages to be followed. The addition of new cells results in migration from the PGZ, through the GZ and TZ. In living lenses, fiber cell differentiation commences at the bottom of the TZ region. Note the hexagonal packing of cells in the TZ at later stages. Cells migrate in the direction of the arrow and accelerate as they approach the equator. At the end of the model run (day 11), four of the original white cells have left the epithelium but some of the white cells remaining in the epithelium have formed clonal groupings.

equator to the anterior pole. Cell density is 2- to 3-fold higher in the GZ than the CZ, suggesting that a large proportion (likely the majority) of the total epithelial population is located within either the GZ or PGZ.

The factors that promote cell proliferation in the PGZ and GZ are not known. A protease-sensitive mitogen was identified in the anterior chamber of the embryonic chicken eye³⁰ and numerous growth factors with potential roles in epithelial cell proliferation are present in the aqueous humor.³¹ Fibroblast growth factor, a growth factor with a demonstrated role in lens fiber cell differentiation,¹⁰ also has been shown, at low concentration, to stimulate lens epithelial cell division, at least in vitro.³² This observation has led to the hypothesis that a posterior-anterior gradient of a single factor, FGF, could serve to trigger both cell division in the GZ and fiber cell formation at the lens equator.³² However, in the present study, two labeling maxima were identified in the GZ. This observation would appear to be inconsistent with the hypothesis that a posterior-anterior gradient of a single factor regulates both differentiation and proliferation.

In other systems, cell proliferation can be influenced by mechanical forces acting on tissues. In that regard, it is interesting to note that in the lens more than 90% of the proliferating cells lie in the region spanned by the anterior and posterior attachment points of the ciliary zonule.³³ The zonule fibers constitute the rigging by which the lens is suspended and centered in the eye. It has been suggested that tension exerted by the zonule could be transduced into a proliferative response,³⁴ although a direct demonstration of this effect is lacking. Interestingly, mutations in fibrillin-1, the principal component of the ciliary zonule, have been shown to underlie Marfan syndrome and Weill Marchesani syndrome. The ocular phenotype for either syndrome can include microspherophakia, a condition in which the lens is abnormally small.

The Penny Pusher Model

The lifelong growth of the lens has prompted some investigators to suggest that a contingent of tissue stem cells might

reside within the epithelium. Based on label-retaining ability and other putative stem cell characteristics, it has been proposed that lens stem cells could be present in the CZ,³⁵ in the region immediately anterior to the GZ,³⁶ in the GZ itself,³⁶ or even in extralenticular locations.³⁷ Although the current data do not exclude the possibility of lens stem cells, they are compatible with a more parsimonious model, in which epithelial cells are of a single type, the behavior of which varies according to latitudinal position only.

We produced a simple physical model (called the *Penny Pusher*, after an arcade game in which players add pennies to the proximal edge of a preexisting sheet of coins with the aim of dislodging, and thereby pocketing, any coins that fall from the distal edge of the sheet) of the lens epithelium based on morphometric and labeling data obtained from 2-month-old mouse lenses (see Figs. 3C, 6). The Penny Pusher was used to model cell production and movement in a sector of the peripheral lens epithelium over an 11-day period (Fig. 10). The assumptions underlying the model were merely that the total number of cells in the epithelium is constant (Fig. 6), cell death is absent (Fig. 9), and S-phase lasts 12 hours.¹⁹ Under these circumstances, introduction of supernumerary cells by mitosis in the GZ or PGZ must result in the displacement of an equal number of TZ cells into the fiber cell compartment. For simplicity, we considered the cell proliferation rate within the GZ (5%) to be uniform, whereas a bimodal distribution of S-phase cells is actually observed (see Figs. 3B, 3C). The proliferative rate in the PGZ was set at 0.5%. To simulate cell division, an appropriate number of new cells (pennies) was introduced randomly into the proliferative compartments (PGZ and GZ) per day. As expected, the introduction of new cells at higher latitudes caused cells at lower latitudes to be displaced toward the edge of the epithelium. The model revealed that cells may accelerate as they approach the equator (see the animated sequence in Supplementary Video S1). Acceleration occurred because of the cumulative displacement effect of mitoses occurring at higher latitudes, which imparted a greater “push” to cells as they approached the equator. Another interesting aspect of the model was that hexagonal packing of

the “cells” was evident in the postmitotic TZ but not in the proliferative GZ and PGZ. In the model, the introduction of new cells into the proliferative regions of the epithelium served to continuously destabilize the hexagonal lattice that would otherwise have formed. Although the pennies in our model bore little physical resemblance to living lens epithelial cells (see Fig. 8A or 8B, for example), it is possible that the need to avoid continuous destabilization of the lattice could be one reason why living lens epithelial cells pass through a postmitotic region (the TZ) before adopting the regular hexagonal packing that characterizes the rest of the fiber cell mass.

By marking random cells in the model (painting them white), we followed the fate of their progeny (Fig. 10). As they were displaced toward the periphery, marked cells were likely to undergo several independent rounds of cell division in the PGZ and GZ and, consequently, clones of labeled cells emerged. The largest clusters developed from cells that were farthest from the equator at the beginning of the model simulation. Those cells had a greater likelihood of undergoing more rounds of mitosis (in the PGZ and subsequently the GZ) than cells that were located close to the equator at the start of the simulation. Over time, the epithelium contained fewer, but larger, clusters of marked cells. The model data thus closely resembled results from lineage-tracing studies in living mouse lenses.¹¹ In those studies, when GFP expression was triggered in a subset of epithelial cells (equivalent to the white pennies in the Penny Pusher model), expression was initially observed in scattered individual cells.¹¹ Over time, however, fewer cell clusters were observed but those nearest the equator contained dozens of cells.

In the Penny Pusher model, cell division was simulated by the random introduction of new cells. In mathematical terms, the model is a realization of a simple branching process with emigration. The Penny Pusher successfully captured important aspects of epithelial cell behavior detected in this and previous studies. However, other characteristics were neglected. For example, in living lenses, the surface area of individual cells varies with latitudinal position and time (Fig. 7) and the anterior lens surface expands significantly as new cells are incorporated into the fiber mass (Fig. 6). We have incorporated these and other features into a rigorous mathematical model of lens growth that relates the production of cells in the epithelium to the deposition of newly differentiated fibers and overall growth of the lens (Sikic H, Shi Y, Lubura S, Bassnett S, unpublished data, 2014). Like the Penny Pusher, the mathematical model belongs to the branching processes family. In comparison, the Penny Pusher is a simple visualization tool. Nevertheless, it provides a satisfying and intuitive explanation for how sustainable flows of cells might be established in the peripheral lens epithelium and how, ultimately, a large group of fiber cells might be derived from a single epithelial cell located near the anterior margin of the PGZ.

Acknowledgments

The authors thank Luka Bassnett for his help in producing the Penny Pusher video sequence.

Supported by National Institutes of Health Grants EY09852 and EY024607 (SB) and P30 EY02687, and an unrestricted grant to the Department of Ophthalmology and Visual Sciences from Research to Prevent Blindness. Supported by a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Programme, FP7-PEOPLE-2013-IOF-622890-MoLeGro (HS) and the Croatian Science Foundation under the project 3526 (SL).

Disclosure: Y. Shi, None; A. De Maria, None; S. Lubura, None; H. Šikić, None; S. Bassnett, None

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